



Investigation of the indigenous fungal community populating barley grains: Secretomes and xylanolytic potential

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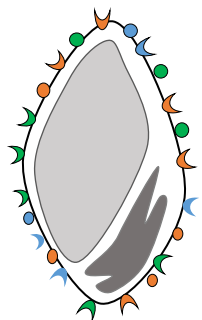
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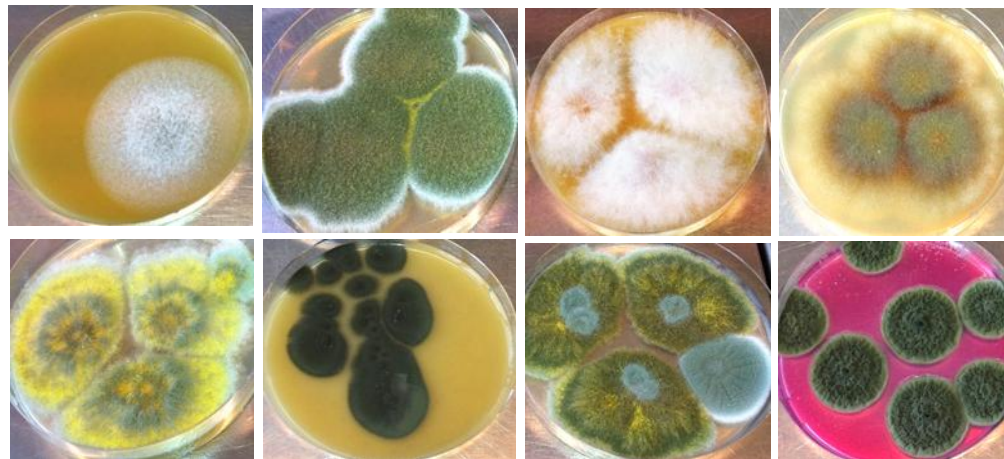
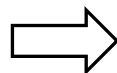
Abstract: The indigenous fungal species populating cereal grains produce numerous plant cell wall-degrading enzymes including xylanases, which could play important role in plant-pathogen interactions and in adaptation of the fungi to varying carbon sources . To gain more insight into the grain surface-associated enzyme activity, members of the populating fungal community were isolated, and their secretomes and xylanolytic activities assessed. Twenty-seven different fungal species were isolated from grains of six barley cultivars over different harvest years and growing sites. The isolated fungi were grown on medium containing barley flour or wheat arabinoxylan as sole carbon source. Their secretomes and xylanase activities were analysed using SDS-PAGE and enzyme assays and were found to vary according to species and carbon source. Secretomes were dominated by cell wall degrading enzymes with xylanases and xylanolytic enzymes being the most abundant. A 2-DE-based secretome analysis of *Aspergillus niger* and the less-studied pathogenic fungus *Fusarium poae* grown on barley flour and wheat arabinoxylan resulted in identification of 82 *A. niger* and 31 *F. poae* proteins many of which were hydrolytic enzymes, including xylanases.

Significance

The microorganisms that inhabit the surface of cereal grains are specialized in production of enzymes such as xylanases, which depolymerize plant cell walls. Integration of gel-based proteomics approach with activity assays is a powerful tool for analysis and characterization of fungal secretomes and xylanolytic activities which can lead to identification of new enzymes with interesting properties, as well as provide insight into plant-fungal interactions, fungal pathogenicity and adaptation. Understanding the fungal response to host niche is of importance to uncover novel targets for potential symbionts, anti-fungal agents and biotechnical applications.



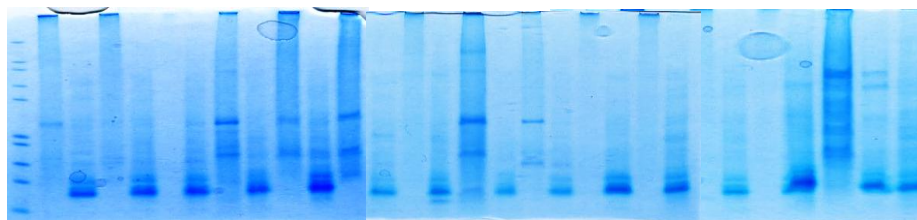
Barley grains



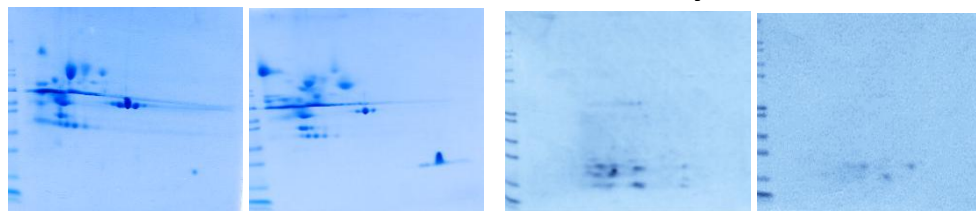
31 fungal isolates



1D secretomes

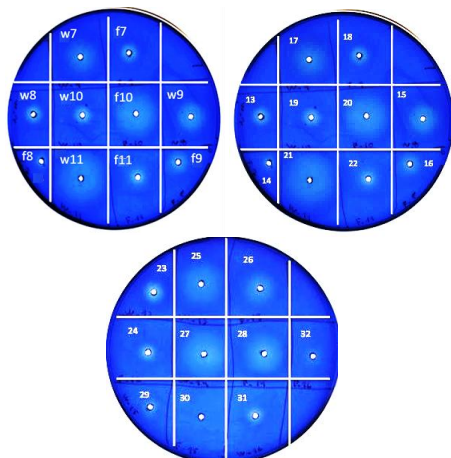


2-DE based secretomics analysis



Identification of cell wall degrading enzymes including xylanases

Qualitative and quantitative
xylanase assays



Highlights

- Profiling the fungal community populating barley grains
- Xylanase production by the indigenous fungi present on barley grains
- Expanding the *Asperigllus niger* secretome
- Initial secretome maps of *Fusarium poae* grown on barley flour and wheat arabinoxylan

Investigation of the indigenous fungal community populating barley grains: secretomes and xylanolytic potential

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Abstract

The indigenous fungal species populating cereal grains produce numerous plant cell wall-degrading enzymes including xylanases, which could play important role in plant-pathogen interactions and in adaptation of the fungi to varying carbon sources . To gain more insight into the grain surface-associated enzyme activity, members of the populating fungal community were isolated, and their secretomes and xylanolytic activities assessed. Twenty-seven different fungal species were isolated from grains of six barley cultivars over different harvest years and growing sites. The isolated fungi were grown on medium containing barley flour or wheat arabinoxylan as sole carbon source. Their secretomes and xylanase activities were analysed using SDS-PAGE and enzyme assays and were found to vary according to species and carbon source. Secretomes were dominated by cell wall degrading enzymes with xylanases and xylanolytic enzymes being the most abundant. A 2-DE-based secretome analysis of *Aspergillus niger* and the less-studied pathogenic fungus *Fusarium poae* grown on barley flour and wheat arabinoxylan resulted in identification of 82 *A. niger* and 31 *F. poae* proteins many of which were hydrolytic enzymes, including xylanases.

Keywords: Environmental proteomics, fungal community, grain proteome, xylanase, barley.

1. Introduction

Over 200 species of microorganisms populate the surface of barley grains, including fungi, yeast and actinomycetes [1]. The composition of the microbial population varies significantly according to developmental stage of the grains, environmental factors, as well as post-harvest storage conditions. The initial colonizers of cereal grains after ear emergence are bacteria, which are replaced by yeasts and eventually by fungi after anthesis [2]. Fungi can have severe effects on the quality of the grains, due to e.g. discoloration, reduced germination, and the production of mycotoxins [3,4]. The fungi colonizing the grains can be categorized as field or storage fungi. The field fungi are those colonizing the developing and mature grains on the plant, with major genera comprising *Alternaria*, *Cladosporium* and *Fusarium*, which typically require high moisture content [3,4]. The storage fungi become more abundant on and within the stored grains, where the moisture level has decreased, these are primarily *Aspergillus* and *Penicillium*. Some of the fungi are known pathogens, e.g. *Fusarium*. Contamination of grains by fungal mycotoxins e.g. aflatoxin and ochratoxin, poses a critical hazard to food safety, human and animal health [5]. A characteristic of the invading fungi is the secretion of a collection of enzymes, including xylanases, polygalacturonases, pectate lyases and lipases, which play important roles in nutrient acquisition, host colonization, virulence and ecological interaction [6,7]. The availability of complete fungal genome sequences and advances in -omics techniques have significantly contributed to a better understanding of plant-fungus interactions, fungal pathogenicity and defense mechanisms in plants. Gel-based proteomics has enabled detailed analysis of several fungal secretomes [8–11]. *Aspergillus oryzae*, traditionally used in production of fermented foods, was found to produce a combination of cell wall degrading enzymes when grown on wheat bran, including β -

glucosidases, α -mannosidases, cellulases and xylanases [12]. The secretome of *A. niger* grown with xylose and maltose was characterized with identification of about 200 proteins and reported to be strongly influenced by the culture conditions and available nutrient source [13]. Secretomes of *F. graminearum* grown on a variety of media, including isolated plant cell walls, wheat and barley flour, contain numerous secreted enzymes such as xylanases, cellulases, proteinases and lipases, depending on the nutrient source [11,14,15]. An obvious key challenge, however, is the further analysis of the function and regulation of the identified fungal proteins, including xylanases.

Although plant responses to fungal attack have been studied mainly in the context of single plant-fungus interactions, plants in the field are exposed to a diverse community of microorganisms, and rely for general protection on proteins and defense molecules produced at the interface with the environment. For example, wheat bran tissues contain numerous oxidative stress and defense-related proteins and inhibitors, as well as proteins that improve tissue strength to hinder pathogen entry [16]. Previously, we investigated the plant-microbe interface by analysis of the surface-associated proteome of barley grains [17] and found this to be dominated by plant proteins with roles in defense and stress-response. However, numerous proteins from the populating microbiota were also identified including fungal and bacterial proteins involved in polysaccharide degradation [17]. Grain-surface-associated xylanase activity was of microbial origin, and xylanases were identified from the fungi *Verticillium dahlia*, *Cochliobolus sativus* (the teleomorph of *Bipolaris sorokiniana*) and *Pyrenophora tritici-repentis* (anamorph of *Drechslera tritici-repentis*) [17], all of which are known to be present on grasses or cereals. To gain more insight into the ability of the fungi to produce xylanases, we isolated and identified fungi from the surface of barley grains

and analysed their secreted proteins and xylanolytic activities. Moreover, to better understand adaptation of the fungi to varying carbon sources, the secretomes of two fungi (*Aspergillus niger* and *Fusarium poae*) grown on barley flour and wheat arabinoxylan to mimic the natural growth substrates, were characterized.

2. Materials and Methods

2.1 Barley grains and growing sites

Barley grains from six cultivars (Barke, Cabaret, Frederik, Quench, Scarlett, Simba) harvested in 2009–2011 were obtained from Sejet Plant Breeding, Horsens, Denmark (9° 50' 51.32" E, 55° 51' 29.27" N, 34 m). Each cultivar was grown in three plots in the same field in a fully randomized block design and grains from different plots were mixed to eliminate location effects. Quench and Simba were also grown at another site in Denmark, Koldkærgaard (10°04' 40.3" E, 56° 18' 28.1" N, 39.99 m) in 2010 and 2011. The cultivar Himalaya harvested in 2003 in Pullman, WA, USA was also analyzed. Danish spring (March–May) and summer (June–August) of 2009 were characterized with mean temperature of 8.3°C and 16.2°C with 652 and 700 hours of sunshine, and 119 and 217 mm of precipitation. Spring and summer of 2011 had mean temperature of 8.1°C and 15.9°C with 301 and 573 hours of sunshine, and 172 and 321 mm of precipitation, respectively (Danish Meteorological Institute). According to the Danish soil classification system, soil at both growing sites Sejet and Koldkærgård were assigned a JB number of five, which denotes a coarse sandy clay texture.

2.2 Isolation of fungi from barley grains

Fungi were isolated and identified by direct plating of 20–35 grains on (i) potato

dextrose agar (PDA [18]), (ii) malt extract agar (MEA) and (iii) MEA Oxoid [19]. For species identification, the isolated fungi were cultivated on a range of different media, including dichloran glycerol (18%) agar (DG18 [20]), dichloran rose bengal yeast extract sucrose agar (DRYES [21]), vegetable juice water (V8 [22]) and Czapek Dox oprodione dichloran agar (CZID [23]). DG18 and DRYES were incubated at 25°C in the dark, while V8 and CZID plates were incubated in alternating light and dark cycles at 20–23°C. For black fungi such as *Alternaria* species, V8, DRYES and potato carrot agar (PCA) were used. For *Fusarium* species, PDA, YES (yeast extract sucrose agar [18]), and SNA (Synthetischer Nährstoffarmer agar) were used. For *Penicillium* species, MEA, YES, CYA (Czapek yeast extract agar [19]), and CREA (Creatine sucrose agar; [18]) were used, while for *Aspergillus* section *Aspergillus* species, CYA, CYA20S (CYA with 20% (w/v) sucrose [19]), CZ (Czapek Dox agar [18]), DG18 and YES, were used. These cultures were incubated for 7 days at different temperatures and alternating dark and light cycles. Fungi were identified based on typical colony form under a stereomicroscope (lower magnification and perception of depth) and conidia morphology with light microscope (higher magnification). The percentage of kernels infected with each identified fungal species was calculated.

2.3 Cultivation of fungi on solid medium

The fungi were cultivated in medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) agar and 1% (w/v) wheat arabinoxylan (WAX) as carbon source [24]. The fungi were grown at 25°C for 7 days.

2.4 Liquid medium

Fungi from densely covered agar plates were used to inoculate 8 mL liquid medium composed of 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) asparagine and 0.5% (w/v) KH_2PO_4 supplemented with either 1% (w/v) WAX or finely ground barley flour as carbon source into 50 mL tubes [25]. The fungi grew on the surface of the medium, and the proteins were secreted into the medium to break down nutrients. Negative controls composed of medium and WAX or barley flour were included. The samples were incubated for 7 days at 25°C. Subsequently, culture supernatants were collected by centrifugation at 3200 g for 30 min at 4°C.

2.5 Washing procedure to extract the surface-associated grain proteins

A washing procedure was implemented that effectively extracts the surface-associated proteins from grains [26] in 25 mM sodium acetate pH 5.0 containing 0.02% (w/v) sodium azide under agitation for 8 h at room temperature. The washing liquids containing extracted proteins were filtered through MN 615 filter paper (Macherey-Nagel, Dueren, Germany) and assayed for xylanase activity.

2.6 Agarose plate assay for detection of xylanase activity

Agarose gels containing dyed substrate (0.1% (w/v) Remazol Brilliant Blue-dyed WAX (Megazyme), 1% (w/v) agarose, 0.2 M sodium citrate-HCl pH 4.8) were prepared in petri dishes. Five microliters of supernatant from fungal liquid cultures were added to 2 mm diameter wells punched into the plates and incubated overnight at room temperature. Xylanase activity appeared as clearing zones around the wells. The assay was used for an initial screen of all fungal isolates grown on barley flour and

WAX, and was subsequently repeated for fungal strains grown on WAX, with similar results (not shown).

2.7 Xylanase activity assay

Xylanase activity was determined in supernatants from fungal liquid cultures using the colorimetric Xylazyme-AX method (Megazyme, Ireland) based on quantification of released products from the azurine-cross linked wheat arabinoxylan (AZCL-AX). Culture supernatants (0.5 mL) were pre-incubated for 10 min at 40°C prior to addition of an AZCL-AX tablet (30% w/v). The mixture was incubated for 30 min at 40°C and 5 mL stop solution (2% (w/v) Tris base pH 9.0) was added and mixed vigorously. After 10 min at room temperature, the reaction mixtures were filtered and the absorbance was measured at 590 nm (Ultrospec II, Amersham Biosciences, Uppsala, Sweden) against a blank prepared by adding 5 mL stop solution to samples prior to addition of substrate. Correction was made for non-enzymatic color release from the AZCL-AX tablets. Duplicate measurements were performed for each of two independent growth experiments.

2.8 Protein content determination and SDS-PAGE

The protein content of the fungal culture supernatants was estimated using the amido black method with bovine serum albumin as standard [27]. Twenty micrograms of protein was precipitated by adding 4 volumes of ice-cold acetone and separated by SDS-PAGE using 4–12% BisTris NuPAGE gels and a vertical slab mini gel unit (NuPAGE Novex system, Invitrogen) according to the manufacturer's instructions. The gels were stained with colloidal Coomassie Blue [28]. A broad-range molecular mass protein ladder (Mark 12TM, Invitrogen) was used.

2.9 2D-gel electrophoresis

Fungal culture supernatants were desalted on a NAP-5 column (GE Healthcare) and 50 µg protein was precipitated by adding four volumes of ice-cold acetone and dissolved in 125 µL rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 200 mM destreak reagent (bis (2-hydroxyethyl) disulfide; GE Healthcare), 0.5% (v/v) pharmalytes pH range 3–10 (GE Healthcare), trace of bromophenol blue). The samples were applied to 7 cm pH 3–10 IPG strips (GE Healthcare) for isoelectric focusing (IEF) (Ettan™ IPGphor; GE Healthcare) after rehydration (12 h at 50 mA/strip at 20°C), performed to reach a total of 20 kVh (1 h at 150 V, 1 h at 300 V, 1 h at 1000 V, gradient to 8000 V, held at 8000 V until a total of 20 kVh). The strips were equilibrated (2 × 15 min) in 5 mL equilibration buffer (6 M urea, 30% (v/v) glycerol, 50 mM Tris HCl, pH 8.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) supplemented with 1% (w/v) dithiothreitol and 2.5% (w/v) iodoacetamide in the first and second equilibration steps, respectively. The strip and molecular weight marker (Mark 12, Invitrogen) were placed on NuPAGE Novex 4–12% Bis-Tris Zoom gels (Invitrogen) and run according the manufacturer's instructions. Gels were stained with colloidal Coomassie Blue (G-250). 2D-gel electrophoresis was performed in duplicate (2 biological replicates, Supplementary Figure S1).

2.10 In-gel digestion and MALDI-TOF/TOF mass spectrometry

Spots or bands were manually excised and subjected to in-gel tryptic digestion [29]. Briefly, gel pieces were washed (100 µL 40% ethanol, 10 min), shrunk (50 µL 100% ACN) and soaked in 2 µL 12.5 ng/µL trypsin (Promega, porcine sequencing grade) in

213 25 mM NH_4HCO_3 on ice for 45 min. The gel pieces were rehydrated by addition of 10
214 μL 25 mM NH_4HCO_3 followed by incubation at 37°C overnight. Tryptic peptides (1
215 μL) were loaded onto an AnchorChipTM target plate (Bruker-Daltonics, Bremen,
216 Germany), covered by 1 μL matrix solution (0.5 $\mu\text{g}/\mu\text{L}$ CHCA in 90% ACN, 0.1%
217 TFA) and washed in 0.5% (v/v) TFA [30]. Tryptic peptides were analyzed on an
218 Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen,
219 Germany) using Flex Control v3.0 and processed by Flex Analysis v3.0 (Bruker-
220 Daltonics, Bremen, Germany). **MS analysis** was performed in positive ion reflector
221 mode with 500 laser shots per spectrum. MS/MS data were acquired with an average
222 of 1000–2000 laser shots for each spectrum. Spectra were externally calibrated using a
223 tryptic digest of β -lactoglobulin (5 pmol/ μL). Internal calibration was performed using
224 trypsin autolysis products (m/z 842.5090, m/z 1045.5637 and m/z 2211.1040).
225 Filtering of spectra was performed for known keratin peaks. Acquired MS and MS/MS
226 spectra were analyzed using Biotoools v3.1 (Bruker-Daltonics, Bremen, Germany).
227 MASCOT 2.0 software (<http://www.matrixscience.com>) was used for database
228 searches in the NCBI nr (National Center for Biotechnology Information) fungi, and
229 green plants (2555264 and 1749148 entries, respectively) and Broad Institute for
230 *Fusarium graminearum* gene index
231 (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum, 13313 entries).
232 The following search parameters were applied: monoisotopic peptide mass accuracy of
233 50 ppm; fragment mass accuracy to ± 0.7 Da; maximum of one missed cleavage;
234 carbamidomethylation of cysteine (fixed) and oxidation of methionine (partial). The
235 signal to noise threshold ratio (S/N) was set to 6. Probability-based MOWSE scores
236 above the calculated threshold value ($p < 0.05$) with a minimum of two matched
237 **unique** peptides were considered for protein identification.

3. Results

3.1. Screening fungal isolates for xylanolytic activity on barley flour and wheat arabinoxylans

To obtain an insight into the plant-fungus relationship, fungal proteins and their enzymatic activities, grain-associated fungi were isolated and identified by direct plating of 20–35 grains on various media and substrates and microscopic examination for subspecies determination. Thirty-one fungal isolates (Table 1) were grown in liquid cultures containing barley flour or WAX as the sole carbon source and the culture supernatants were analyzed for xylanase production.

Table 1. Fungal isolates used for xylanase activity assays.

Fungal Isolate	Species	Barley cultivar	Harvest Year
1	<i>Fusarium avenaceum</i>	Quench	2010
2	<i>Fusarium avenaceum</i>	Quench	2011
3	<i>Fusarium avenaceum</i>	Quench	2010
4	<i>Fusarium culmorum</i>	Quench	2011
5	<i>Fusarium graminearum</i>	Simba	2010
6	<i>Acremonium verrucosum</i>	Cabaret	2011
7	<i>Epicothium nigrum</i>	Scarlett	2009
8	<i>Fusarium poae</i>	Frederik	2009
9	<i>Fusarium equiseti</i>	Quench	2010
10	<i>Drechslera</i> sp.	Scarlett	2011
11	<i>Alternaria infectoria</i>	Simba	2011
12	<i>Epicothium nigrum</i>	Simba	2010
13	<i>Alternaria tenuissima</i>	Scarlett	2009
14	<i>Alternaria infectoria</i>	Frederik	2009
15	<i>Cladosporium</i> sp.	Scarlett	2009
16	<i>Drechslera</i> sp.	Quench	2010
17	<i>Cladosporium</i> sp.	Quench	2010
18	<i>Cladosporium</i> sp.	Frederik	2009
19	<i>Penicillium brevicompactum</i>	Simba	2011
20	<i>Penicillium brevicompactum</i>	Quench	2010
21	<i>Penicillium brevicompactum</i>	Cabaret	2011
22	<i>Penicillium verrucosum</i>	Cabaret	2011
23	<i>Aspergillus niger</i>	Simba	2010
24	<i>Penicillium chrysogenum</i>	Scarlett	2011
25	<i>Aspergillus pseudoglaucus</i>	Simba	2010
26	<i>Aspergillus pseudoglaucus</i>	Scarlett	2011

27	<i>Phoma</i> sp.	Cabaret	2009
28	<i>Fusarium tricinctum</i>	Cabaret	2009
29	<i>Fusarium tricinctum</i>	Cabaret	2009
30	<i>Penicillium cyclopium</i>	Himalaya	2003
31	<i>Penicillium freii</i>	Himalaya	2003

WAX was used to induce the production of xylan degrading enzymes, while barley flour was used to resemble a natural substrate for the fungi. Plate zymograms prepared with 0.1% dyed substrate (RBB WAX) were used as a screen for xylanolytic activity (Fig.1). Xylanases produced by the cultivated fungi could be assessed qualitatively by the clarity and size of the degradation zones surrounding the punched wells.

The culture supernatants from WAX showed more prominent clearing zones compared to barley flour. Noticeably, the storage fungi *Penicillium* and *Aspergillus* gave rise to larger and more opaque degradation zones, while field fungi such as *Fusarium*, *Alternaria*, *Epicoccum* and *Drechslera* spp. generally gave rise to smaller and clearer zones. The different qualitative character of the clearing zones will depend on the amount and activity of the xylanase(s) present in the samples and be influenced by different growth rates of the fungi in the liquid cultures, but may also reflect production of xylanases with different functional characteristics. Quantitative xylanase activity assays were performed for five selected fungi of three genera (*Fusarium*, *Phoma* and *Penicillium*, Table 2) isolated from the two barley cultivars Cabaret 2009 and Himalaya 2003, which exhibited high (0.127 U/g) and low (0.043 U/g) surface-associated xylanase activity levels. The field fungus species *F. tricinctum* and *Phoma* spp. were predominantly found on Cabaret 2009, while storage fungus species *P. cyclopium* and *P. freii* essentially found on Himalaya 2003.

Table 2. Xylanase activity measured in culture supernatants from **five selected** fungi grown in medium with wheat arabinoxylan and ground barley flour as substrate.

Fungal isolate	Species	Specific xylanase activity (U/g)			
		Replicate 1		Replicate 2	
		WAX	Flour	WAX	Flour
27	<i>Phoma</i> sp.	0.01	0.70	0.01	0.95
28	<i>Fusarium tricinctum</i>	0.18	0.96	0.15	0.92
29	<i>Fusarium tricinctum</i>	0.40	5.60	0.15	2.91
30	<i>Penicillium cyclopium</i>	1.52	7.77	1.03	1.82
31	<i>Penicillium freii</i>	3.25	290.72	5.79	366.99
	Negative control	0.01	0.00		

3.2. Secreted protein profiles of **five selected** fungi grown on barley flour and wheat arabinoxylans

SDS-PAGE was used to screen the secreted protein profiles of the fungal isolates grown on WAX and barley flour (Fig. 2). Protein patterns of the cultivated fungi are species-specific, reflecting secretion of different proteins. The fungal supernatants grown with barley flour as carbon source displayed a prominent band of molecular size of 10 kDa, which is probably a barley protein originating from the medium. The fungal strains grown with WAX gave rise to faint bands after Coomassie Blue staining and an accumulation of high molecular weight material was visible in the wells. Twenty bands (Fig. 2) of **five selected fungi** were excised, tryptic digested and analyzed by **MALDI-TOF/TOF**, which resulted in ten confident identifications, of which four (bands 3, 8, 14 and 15) were identified as endo-1,4- β -xylanases (Table 3). Other bands contained cell wall-degrading enzymes and hypothetical proteins.

Table 3. Identification of proteins in SDS-PAGE bands of culture supernatants from **five selected** fungi grown on WAX and barley flour^a.

no ^b	Accession no.	Organism	Protein (GH family)	Mw theor/ meas	PMF score	E-value	Sequence coverage %	Unique peptides	MS/MS Precursor ions	MS/MS Peptide sequences	Ion score	Expect
2	gi 255931857	<i>Penicillium chrysogenum</i>	Alpha-amylase GH13	51032/ 68700	179	1.9E-12	14	5	1112.5729 1830.8534 2312.0883	R.NIYFALTDR.I R.DLYSINENYGTADDLK.S R.GIPIVYYGTEQGYAGGNP ANR.E	53 61 36	0.0089 0.0012 0.33
3	gi 169159203	<i>Penicillium citrinum</i>	Endo-1,4-beta-xylanase GH11	35338/ 38000	134	4.9E-08	24	7	934.5074 807.4367	R.VIGEDFVR.I R.IAFETAR.A	53 40	0.014 0.24
4	gi 70996610	<i>Aspergillus fumigatus</i>	Beta-xylosidase XylA GH3	86731/ 121600	250	1.2E-19	13	9	1612.8372 1778.9684 892.4683	R.YGLDVYAPNINAFR.S R.VLYPGKYELALNNER.S K.WLVGFDR.L	89 87 40	2.3e-06 3.00E-06 0.29
6	gi 255930951	<i>Penicillium chrysogenum</i>	Endo-arabinase GH43	36188/ 33600	118	1.80E-06	15	3	1795.958 1676.7563	K.VGADGVTPIGDAVQILDR.D K.TGLISPGGGNVCGCGDR.M	62 28	0.00086 2.3
8	gi 3915310	<i>Aspergillus aculeatus</i>	Endo-1,4-beta-xylanase GH10	35423/ 38000	156	3.10E-10	15	4	807.434 1775.9111	R.IAFETAR.A K.LYINDYNLDSASYPK.L	43 90	0.12 1.60E-06
9	gi 344228869	<i>Candida tenuis</i>	Hypothetical protein	76355/ 113600	76	0.031	16	8				
11	gi 310699603	<i>Fusarium oxysporum</i>	Alpha-D-galactopyranosidase GH31	45222/ 46600	94s	0.00055	14	5	984.4848	K.FGLYGDGGAK.T	59	0.0021
14	gi 374253734	<i>Fusarium oxysporum</i>	Endo-1,4-beta-xylanase A GH10	36403/ 48600	95	0.00038	13	4	935.541 1155.6408	R.LVKSYGRL.I K.QYFGTALTVR.N	40 26	0.19 6.4
15	gi 302913666	<i>Nectria haematococca</i>	Hypothetical protein (xylanase GH11)	24103/ 32600	65	0.35	17	3	1989.9925	K.KGEVTVDGSVYDIYTSTR.T	46	0.035
20	gi 119481903	<i>Neosartorya fischeri</i>	Alpha-L-arabinofuranosidase A GH43	70046/ 75100	100	0.00014	3	2	1561.7308	R.FPGGNNLEGDTIDGR.W	93	9.00E-07

^a theor: theoretical; meas.: measured; GH: glycoside hydrolase

^b refers to Fig. 2.

3.3 Profiling the fungal communities on barley grains

Barley harvested from two different sites in years 2009–2011 was used to profile the fungal communities. Surface xylanase activity [17] of the grains was measured in parallel (Table 4). The barley samples could be divided into two groups with low (<0.06 U/g) and high (>0.10 U/g) activity, respectively. The two most predominant fungal species, identified on the majority of grain samples were *Alternaria infectoria* and *Fusarium culmorum*. The cultivars (cvs.) showed varying levels of grain colonization by different fungal species. The predominant species harboured by the low xylanase group comprising cvs. Frederik, Simba, Barke, Cabaret, Quench (all 2011), Cabaret (2010) and Himalaya (2003), were *Aspergillus pseudoglaucus*, *Epicoccum nigrum*, *Penicillium spathulatum* and *Chalastospora gossypii* (Table 4). The high xylanase group comprising Cabaret, Frederik and Simba (all 2009), Quench and Simba (2011) contained *F. tricinctum*, *Cochiobolus sativus* (Drechslera), *Gonatobotrys simplex* and *Phoma* sp. However, there was no clear correlation between fungal species, growth location or year, and grain surface xylanase activity.

3.4 Proteome analysis of *Aspergillus niger* and *Fusarium poae* secretomes on barley flour and wheat arabinoxylan

To access the secretomes of *A. niger* (isolate 23) and *F. poae* (isolate 8), 2-DE was performed to map and identify the proteins in the culture medium containing WAX and barley flour. Representative 2D-gels (pH 4–8.5) of the secretomes of *A. niger* and *F. poae* grown on WAX and barley flour are shown (Fig. 3). The protein patterns of *A. niger* grown either on WAX or barley flour were similar and contained approximately 105 resolved spots, while the *F. poae* secretomes were less well resolved and with

only 54 spots. Visible spots were excised from the 2D gels for identification by
MALDI-MS and MS/MS, which resulted in 82 and 30 confident protein identifications
from the *A. niger* and *F. poae* gels, respectively (Table 5, Supplementary Table S1).
The identified proteins included glycoside hydrolases, proteases, oxidoreductases,
esterases, nucleases, lyases, housekeeping enzymes, hypothetical proteins, and
proteins with unknown function.

31 **Table 4.** The isolated fungi listed in order of incidence (number of samples from which the fungus was isolated) followed by severity
 32 (percentage of grains containing fungus).

Cultivar ^a	Frederik	Simba	Himalaya	Barke	Cabaret	Cabaret	Quench	Cabaret	Frederik	Quench	Simba	Simba
Growth location ^b	S	S	U	S	S	S	S	S	S	K	S	K
Harvest year	2011	2011	2003	2011	2011	2010	2011	2009	2009	2011	2009	2011
Surface xylanase (U/g)	0.031	0.041	0.043	0.057	0.058	0.059	0.060	0.127	0.168	0.171	0.171	0.172
Percentage of grains with fungus ^c (incidence)												
<i>Alternaria infectoria</i> (10)	40	5	-	2	10	7	30	-	40	5	40	5
<i>Fusarium culmorum</i> (8)	7	4	-	15	7	17	30	-	-	40	-	40
<i>Epicoccum nigrum</i> (7)	-	20	14		5	-	1	-	-	5	10	2
<i>Aspergillus pseudoglaucus</i> (6)	30	-	5	4	12	-	-	-	2	-	-	1
<i>Nigrospora spp.</i> (5)	2	-	-	2	30	-	-	-	-	-	2	30
<i>Gonatobotrys simplex</i> (5)	2	-	-	2	-	-	-	-	20	2	20	-
<i>Cochliobolus sativus (Drechslera)</i> (5)	-	15	-	2	2	7	2	-	-	20	-	20
<i>Penicillium spathulatum</i> (3)	4	4	-	40	-	-	-	-	-	-	-	-
<i>Chalastospora gossypii (Alt. malorum)</i> (2)	-	-	28	-	-	7	-	-	-	-	-	-
<i>Cladosporium cladosporioides (complex)</i> (2)	-	15	-	-	-	-	-	-	-	5	-	-
<i>Aspergillus spinulosporus</i> (2)	10	-	-	-	-	-	-	-	5	-	-	-
<i>Harzia vernicosa</i> (2)	-	-	-	-	-	-	-	-	5	-	-	2
<i>Tricoderma hamatum</i> (2)	4	-	-	-	-	-	-	-	2	-	-	-
<i>Phoma sp.</i> (1)	-	-	-	-	-	-	-	50	-	-	-	-
<i>Fusarium tricinctum</i> (1)	-	-	-	-	-	-	-	25	-	-	-	-
<i>Claetomium globosum</i> (1)	-	20	-	-	-	-	-	-	-	-	-	-
<i>Penicillium scabrosum</i> (1)	-	15	-	-	-	-	-	-	-	-	-	-
<i>Fusarium poae</i> (1)	-	-	-	-	-	-	-	-	5	-	-	-
<i>Penicillium verrucosum</i> (1)	-	-	-	-	-	-	5	-	-	-	-	-
<i>Acremoniella verrucosa</i> (1)	-	-	-	-	5	-	-	-	-	-	-	-
<i>Penicillium brevicompactum</i> (1)	-	-	-	-	5	-	-	-	-	-	-	-
<i>Penicillium verrucosum</i> (1)	-	-	-	-	5	-	-	-	-	-	-	-
<i>Rhizopus nigricans</i> (1)	-	-	5	-	-	-	-	-	-	-	-	-
<i>Penicillium freii</i> (1)	-	-	5	-	-	-	-	-	-	-	-	-
<i>Wallemia</i> (1)	-	-	-	4	-	-	-	-	-	-	-	-
<i>Ulocladium atrium</i> (2)	-	-	15	-	-	-	-	-	2	-	-	-
<i>Penicillium sp.</i> (1)	-	-	-	-	-	-	-	-	-	-	-	2

33 ^a Barley samples listed in order of increasing surface xylanase activity

34 ^b S: Sejet, Denmark; U: Pullman, WA, USA; K: Koldkærsgaard, Denmark

35 ^c Fungi listed in order of incidence (number of samples from which the fungus was isolated) followed by severity (percentage of grains containing fungus)

36

37 **Table 5. MALDI-MS** and MS/MS identification of proteins from culture supernatants of *Aspergillus niger* and *Fusarium poae* grown
 38 with wheat arabinoxylan (WAX) or barley flour as carbon source. Spot numbers correspond to Fig.3. For identification details refer to
 39 Supplementary Table S1.

Spot no.	Accession	Locus	Organism	Protein name
<i>Aspergillus niger</i> on barley flour				
a1	gi 358375153	GAA91739	<i>Aspergillus kawachii</i> IFO 4308	Extracellular α -glucosidase (AgIU) GH31
a2	gi 224027	1008149A	<i>Aspergillus niger</i>	Glucoamylase G1 GH15
a4	gi 3913152	AXHA_ASPTU	<i>Aspergillus tubingensis</i>	Alpha-L-arabinofuranosidase axhA GH43_62_32_68
a5	gi 3913152	AXHA_ASPTU	<i>Aspergillus tubingensis</i>	Alpha-L-arabinofuranosidase axhA GH43_62_32_68
a6	gi 317028138	XP_001389996	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase F1 GH10
a8	gi 358375979	GAA92552	<i>Aspergillus kawachii</i> IFO 4308	Endo-1,4- β -xylanase A
a10	gi 317028138	XP_001389996	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase F1 GH10
a11	gi 1362263	S55931	<i>Aspergillus niger</i>	Cellulase GH12
a12	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a13	gi 1362263	S55931	<i>Aspergillus niger</i>	Cellulase GH12
a14	gi 1362263	S55931	<i>Aspergillus niger</i>	Cellulase GH12
a15	gi 1362263	S55931	<i>Aspergillus niger</i>	Cellulase GH12
a16	gi 19919756	AF490982_1	<i>Aspergillus niger</i>	Endo-1,4- β -xylanase GH11
a19	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a20	gi 19919756	AF490982_1	<i>Aspergillus niger</i>	Endo-1,4- β -xylanase A GH11
a21	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a22	gi 9858848	AAG01166	<i>Aspergillus niger</i>	Xylanase GH11
a23	gi 19919756	AF490982_1	<i>Aspergillus niger</i>	Endo-1,4- β -xylanase GH11
a24	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a25	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a30	gi 358369412	GAA86026	<i>Aspergillus kawachii</i> IFO 4308	Hypothetical protein AKAW_04140 Anti-fungal protein
a31	gi 328864038	EGG13137	<i>Melampsora larici-populina</i> (strain 98AG31)	Hypothetical protein Melladraft_58483 Putative uncharacterized protein

a64	gi 317025187	XP_001388626	<i>Aspergillus niger</i> CBS 513.88	Xyloglucanase
a65	gi 358369379	GAA85994	<i>Aspergillus kawachii</i> IFO 4308	Beta-mannosidase (MndA)
a68	gi 358370052	GAA8666	<i>Aspergillus kawachii</i> IFO 4308	Beta-galactosidase (LacA) GH35
a69	gi 461623	BGAL_ASPNG	<i>Aspergillus niger</i>	Beta-galactosidase GH35
a70	gi 134077473	P29853	<i>Aspergillus niger</i>	Alpha-mannosidase GH92
a71	gi 350633946	EHA22310	<i>Aspergillus niger</i> ATCC 1015	Hypothetical protein ASPNIDRAFT_50997
a72	gi 358373696	AGALC_ASPNG Q9UUZ4	<i>Aspergillus kawachii</i> IFO 4308	Alpha-galactosidase C GH36
a73	gi 358375222	GAA91807	<i>Aspergillus kawachii</i> IFO 4308	Mycelial catalase Cat1
a74	gi 358375153	GAA91739	<i>Aspergillus kawachii</i> IFO 4308	Extracellular α -glucosidase (AgIU) GH31
a75	gi 358375153	GAA91739	<i>Aspergillus kawachii</i> IFO 4308	Extracellular α -glucosidase (AgIU) GH31
a76	gi 358370756	GAA87366	<i>Aspergillus kawachii</i> IFO 4308	Alpha-glucosidase GH31
a77	gi 358370756	GAA87366	<i>Aspergillus kawachii</i> IFO 4308	Alpha-glucosidase GH31
a78	gi 358368862	GAA85478	<i>Aspergillus kawachii</i> IFO 4308	Alpha-1,3-glucanase/mutanase GH71
a79	gi 350633883	EHA22274	<i>Aspergillus niger</i> ATCC 1015	Hypothetical protein ASPNIDRAFT_123586
a80	gi 350633910	EHA22274	<i>Aspergillus niger</i> ATCC 1015	XynE_like Hypothetical protein ASPNIDRAFT_54865
a81	gi 145231236	XP_001389882	<i>Aspergillus niger</i> CBS 513.88	Fatty_acyltransferase_like
a82	gi 358367957	GAA84575	<i>Aspergillus kawachii</i> IFO 4308	Phospholipase C PLC-C
a83	gi 317036371	XP_001398198	<i>Aspergillus niger</i> CBS 513.88	Cholinesterase
a84	gi 358365618	GAA82240	<i>Aspergillus kawachii</i> IFO 4308	Carboxypeptidase S1
a85	gi 308212489	ADO21450	<i>Aspergillus niger</i>	Carboxypeptidase CpdS
a86	gi 350631148	EHA19519	<i>Aspergillus niger</i> ATCC 1015	Exoglucanase CBHII GH7
a87	gi 189484494	ACE00420	<i>Aspergillus niger</i>	Alpha-amylase A GH13
a88	gi 74698498	CBHB_ASPNG	<i>Aspergillus niger</i>	Alpha-L-arabinofuranosidase E
a89	gi 157829865	1AGM_A	<i>Aspergillus Awamori</i> Var. X100	Beta-D-glucan cellobiohydrolase B GH7
a90	gi 55670667	1WD3_A	<i>Aspergillus kawachii</i>	Chain A, Refined structure for the complex of acarbose with glucoamylase Chain A, Crystal structure of arabinofuranosidase

***Aspergillus niger* on WAX**

a32	gi 40313280	BAD06004	<i>Aspergillus awamori</i>	Glucoamylase, GH15
a33	gi 358375978	GAA92551	<i>Aspergillus kawachii</i> IFO 4308	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68
a34	gi 259016351	FAEA_ASPAW	<i>Aspergillus awamori</i>	Feruloyl esterase A
a35	gi 3913152	AXHA_ASPTU	<i>Aspergillus tubingensis</i>	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68
a36	gi 3913152	AXHA_ASPTU	<i>Aspergillus tubingensis</i>	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68
a37	gi 300706143	XP_002995371	<i>Nosema ceranae</i> BRL01	Hypothetical protein NCER_101765

a38	gi 254571817	XP_002493018	<i>Komagataella pastoris</i> GS115	Hypothetical protein
a39	gi 317028138	XP_001389996	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase F GH10
a42	gi 358375979	GAA92552	<i>Aspergillus kawachii</i> IFO 4308	Endo-1,4- β -xylanase A GH11
a43	gi 317028138	XP_001389996	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase F1 GH10
a44	gi 1362263	S55931	<i>Aspergillus niger</i>	Cellulase GH12
a45	gi 19919756	AF490982_1	<i>Aspergillus niger</i>	Endo-1,4- β -xylanase GH11
a46	gi 380865431	XYNB_ASPKW	<i>Aspergillus kawachii</i> IFO 4308	Endo-1,4- β -xylanase B GH11
a47	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a48	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a49	gi 13242071	AAK16546	<i>Aspergillus niger</i>	Xylanase
a50	gi 13242071	AAK16546	<i>Aspergillus niger</i>	Xylanase
a51	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a52	gi 19919756	AF490982_1	<i>Aspergillus niger</i>	Endo-1,4- β -xylanase, GH11
a56	gi 9858848	AAG01166	<i>Aspergillus niger</i>	xylanase GH11
a63	gi 145228427	XP_001388522	<i>Aspergillus niger</i> CBS 513.88	Endo-1,4- β -xylanase A GH11
a89	gi 189484494	ACE00420	<i>Aspergillus niger</i>	Alpha-L-arabinofuranosidase E GH43
a90	gi 189484494	ACE00420	<i>Aspergillus niger</i>	Alpha-L-arabinofuranosidase E GH43
	gi 145234270	XP_001400506	<i>Aspergillus niger</i> CBS 513.88	Glutaminase GtaA
a91	gi 358370298	GAA86910	<i>Aspergillus kawachii</i> IFO 4308	Six-hairpin glycosidase
a92	gi 134083763	CAK47097	<i>Aspergillus niger</i>	Peptidase_S10
a93	gi 358370493	GAA87104	<i>Aspergillus kawachii</i> IFO 4308	Melibiose D (GH27 or 13)
a94	gi 4235093	AAD13106	<i>Aspergillus niger</i>	Beta-xylosidase GH3
a95	gi 7009581	CAB75696	<i>Aspergillus niger</i>	Beta-glucosidase
a96	gi 118582212	ABL07484	<i>Aspergillus niger</i>	Lactase, partial
a97	gi 358367698	GAA84316	<i>Aspergillus kawachii</i> IFO 4308	Hypothetical protein AKAW_02431
a98	gi 358376345	GAA92905	<i>Aspergillus kawachii</i> IFO 4308	Beta-glucosidase
99	gi 358373696	GAA90293	<i>Aspergillus kawachii</i> IFO 4308	Alpha-galactosidase C
a100	gi 358375222	GAA91807	<i>Aspergillus kawachii</i> IFO 4308	Mycelial catalase Cat1
a101	gi 3912991	AGUA_ASPTU O42814	<i>Aspergillus tubingensis</i>	Alpha-glucuronidase A GH67
a102	gi 358375006	GAA91593	<i>Aspergillus kawachii</i> IFO 4308	Alpha-xylosidase GH31
103	gi 358370259	GAA86871	<i>Aspergillus kawachii</i> IFO 4308	EstA precursor
	gi 358370442	GAA87053	<i>Aspergillus kawachii</i> IFO 4308	Tripeptidyl-peptidase
a104	gi 55670667	1WD3_A	<i>Aspergillus kawachii</i>	Chain A, Crystal structure of arabinofuranosidase
a105	gi 358367805	GAA84423	<i>Aspergillus kawachii</i> IFO 4308	Beta-glucuronidase

***Fusarium poae* on barley flour**

f2	gi 1310677	CAA66232	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Protein z-type serpin
f3	gi 1310677	CAA66232	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Protein z-type serpin

f4	gi 1310677	CAA66232	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Protein z-type serpin
f5	gi 1310677	CAA66232	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Protein z-type serpin
f11	gi 46120810	XP_385112	<i>Gibberella zeae</i> PH-1	Hypothetical protein FG04936.1
				Aminopeptidase Y
f12	gi 358375979	GAA92552	<i>Aspergillus kawachii</i> IFO 4308	Endo-1,4- β -xylanase A GH10
f23	gi 225102	1208404A	<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>	Trypsin/amylase inhibitor pup13
f24	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f25	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f26	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f27	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f28	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f29	gi 585290	IAAB_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase/trypsin inhibitor CMb
f30	gi 225102	1208404A	<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>	Trypsin/amylase inhibitor pUP13
f31	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f32	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f33	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f34	gi 149237516	XP_001524635	<i>Lodderomyces elongisporus</i> NRRL YB-4239	Ubiquitin
f36	gi 1405736	CAA35188	<i>Hordeum vulgare</i>	Trypsin inhibitor cme precursor
f37	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
f38	gi 123970	IAA2_HORVU	<i>Hordeum vulgare</i>	Alpha-amylase inhibitor BDAI-1
	gi 326503930	BAK02751	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Predicted protein
				Ubiquitin
f39	gi 1405736	CAA35188	<i>Hordeum vulgare</i>	Trypsin inhibitor cme precursor
f40	gi 68305063	BAK02751	<i>Triticum aestivum</i>	Ubiquitin, partial
	gi 1588926	2209398A	<i>Triticum aestivum</i>	Pathogenesis-related protein
f41	gi 1405736	CAA35188	<i>Hordeum vulgare</i>	Trypsin inhibitor cme precursor
f44	gi 326499596	BAJ86109	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Predicted protein, Gamma-thionin
f45	gi 225465030	XP_002265864	<i>Vitis vinifera</i>	Ubiquitin-NEDD8-like protein RUB2
f46	gi 326503930	BAK02751	<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Predicted protein
				Ubiquitin
<i>Fusarium poae</i> on WAX				
f48	gi 46115498	XP_383767	<i>Gibberella zeae</i> PH-1	Hypothetical protein FG03591.1
				GH18_chitinase
f50	gi 46138441	XP_390911	<i>Gibberella zeae</i> PH-1	Hypothetical protein FG10735.1
f52	gi 46138969	XP_391175.1	<i>Gibberella zeae</i> PH-1	Hypothetical protein FG10999.1
				Endo-1,4- β -xylanase GH11

4. Discussion

4.1. Profiling the populating fungal community on barley grains and their secretomes

Several proteomics studies have analyzed the effect of fungal infections on cereal grain proteomes [31,32]. Metagenomics and metaproteomics analyses of the populating microbial communities and proteomes have to date mainly been applied to the rhizosphere and phyllosphere [33]. Little is yet known about the dynamic composition of the microbial populations on barley grains, their interactions with the host, proteomes and enzymatic activities. In this study, barley cvs. over different harvest years and growing sites were cultivated in a range of different media to compare the composition of the fungal populations, and to investigate their secretomes and xylanolytic activities. In general the grains were colonized by field fungal genera *Alternaria*, *Fusarium*, *Dreschlera* (*Cochliobolus sativus*) and *Nigrospora* and *Epicoccum*, which are all known to be part of the natural mycobiota on cereals. The universal presence of *Alt. infectoria* on barley grains has also been reported elsewhere [4]. Identification of high numbers of field fungi on stored grains from the 2009 harvest indicates that field fungal spores survived the storage period and still provide a snapshot of conditions in the field. In a previous study of the surface-associated proteome of barley grains, among the identified microbial proteins, numerous fungal proteins were found [17]. The matched database sequences originated from the fungal genera *Dreschlera*, *Fusarium* and *Penicillium*, all of which were isolated and identified in the present study. The identified proteins included xylanases of glycoside hydrolase families GH10 and GH11 from *Cochliobolus sativus* (*Dreschlera sorokiniana* = *Bipolaris sorokiniana*) and *Pyrenophora tritici-repentis* (teleomorph of

Drechslera tritici-repentis) and enzymes involved in primary metabolism such as glyceraldehyde 3-phosphate dehydrogenase from *Fusarium* and RNA processing protein IPI3 from *Penicillium* [17]. The correlation between the inferred origin of identified fungal proteins in the washing liquids of barley grains with the current set of isolated fungi further validates the approach.

The presence of fungal species on barley grains is highly dependent on environmental factors. In samples from 2009, where there was a warm spring and summer in Denmark (see 2.1), high numbers of the genera *Alternaria*, *Fusarium*, *Gonatobotrys* and *Phoma* are seen (Table 4). In contrast, spring and summer of 2011 had fewer hours of sunshine and more precipitation (see 2.1) and samples are dominated by *Alternaria*, *Fusarium* and *Aspergillus* section *Aspergillus*. In addition, the barley cultivar Himalaya harvested in the US in 2003 was analyzed and exhibited a distinct profile with *Chalastospora gossypii* (earlier name *Alt. malorum*), *Ulocladium atrum* and *Epicoccum nigrum*. Barley cvs. harvested in UK were reported to be invaded by large numbers of *Alt. alternate*, *Cladosporium Cladosporioides*, *Aureobasidium pullulans*, *Epicoccum nigrum*, and several *Penicillium* species [2]. Most of the barley-associated fungi are regarded as non-toxicogenic including *Cladosporium*, *Aspergillus pseudoglaucus* and *Epicoccum*. Both *Alternaria* and *Cladosporium* have been reported to cause various degrees of grey discoloration, which in part can be due to pigments in their mycelium or from melamins produced by plant cells. However, some fungi not only cause staining to the grains, but also produce mycotoxins, which are harmful to humans and animals [4,34]. High levels of *Fusarium* can be harmful, as some species are capable of producing an array of mycotoxins [4,18]. Other commonly known toxicogenic species include *Alternaria* (except *Alt. infectoria* species-group), *Aspergillus* and *Penicillium verrucosum* (ochratoxin A and citrinin) and *P. freii* (xanthomegnin

and viomellein).

No clear correlation was observed between any of the isolated fungal species and the surface xylanase activity measured for the corresponding samples (Table 1 and 4).

This probably reflects the contribution of multiple organisms to the total xylanolytic capacity of the population, many of which may not be culturable and are therefore not taken into account using the present methodology.

4.2 Profiling the grain-associated fungal secretomes and xylanolytic activities

Fungi colonizing the grains must be able to proliferate and become established rapidly, as well as to produce necessary enzymes for nutrient acquisition. Fungal growth is strongly influenced by abiotic factors such as temperature, water activity and pH. In this study, fungal isolates were grown in WAX and barley flour and culture supernatants analyzed for xylanolytic activities. It might be expected that the fungi would produce higher xylanase activity levels when grown on WAX compared to barley flour, since the amount of arabinose (36%) and xylose (51%) is much higher in WAX than in barley flour, which is a complex mixture of nutrients, including starch, non-starch polysaccharides (e.g. β -glucans), proteins and lipids. Arabinoxylans constitute about 7.1–8.0% of barley grains [35]. Moreover, barley grains contain xylanase inhibitors targeting bacterial and fungal xylanases [36], which would decrease the amount of measureable xylanase activity in flour-containing culture supernatants. While this is suggested by smaller or weaker clearing zones produced by supernatants from flour-grown cultures (Fig. 1), it is not the case for all and the higher xylanase for isolates 27–31 is for flour cultures (Table 2), probably reflecting superior fungal growth supported by the more complex flour medium.

The storage fungi genera *Penicillium* and *Aspergillus* are known to produce higher xylanase levels than the field fungi [37–39]. In the field, invading fungi can infect the grain and further invade the plant, while storage fungi only have access to the grain as source of nutrition. The storage fungi are therefore more specific in nutrient acquisition [37,40], which might explain their higher xylanase production.

Analysis of SDS-PAGE bands from culture supernatants enabled identification of proteins with roles in degradation of complex polysaccharides. Synergistic actions of the identified arabinases, xylanases and xylosidases can efficiently depolymerize the arabinoxylan found in high abundance in plant cell walls, thus drastically changing the mass and solubility of the substrate enhancing nutrient availability. Four of the ten identified proteins were xylanases, validating the approach for identification of xylanolytic activities from grain-associated fungi. Notably, none were identical to the xylanases identified directly from the grain surface proteome [17], indicating the complementary of the approaches applied. Two xylanases from family GH10 and two from GH11 were identified (Table 3). Based on these identifications it was decided to undertake a 2-DE-based analysis of secretomes from two selected fungal isolates.

4.3 Profiling the 2D-secretome of *Aspergillus niger* grown on barley and WAX

Proteomes are dynamic, and a large amount of information about the functional responses of an organism can be obtained by characterizing the proteome under different physiological conditions. Hitherto, only a few proteomics studies have been performed on grain-associated fungi with substrates present in the host plant [11,14,41,42]. Secretome analysis of *A. niger* grown on WAX and barley flour as sole

137 carbon source resulted in identification of a battery of proteins targeted towards plant
 138 cell wall degradation and carbohydrate catabolism. The majority of the identifications
 139 were of xylanolytic enzymes, namely 1,4- β -arabinoxylan arabinofuranohydrolase
 140 AxA (GH43, spots 4–5, 33, 35–36 and 62), α -L-arabinofuranosidase A and E (GH51,
 141 spots 51, 86 and GH43, spot 89), α -glucuronidase A (GH67, spot 101), β -
 142 glucuronidase (GH2, spot 105), endo-1,4- β -xylanases F1 (GH10, spots 6, 10, 39, 43),
 143 A (GH11, spots 8, 12, 19, 21, 24–25, 42, 47–48, 51 and 63) and B (GH11, spot 46),
 144 xyloglucanase (GH16, spot 64), and α - and β -xylosidase (GH31, spot 102 and GH3,
 145 spot 94). α -Galactosidase C and D (melibiase, GH36, spots 72, 99 and GH27, spot 93),
 146 and β -galactosidase (LacA, GH35, spot 68 and 69) found from *A. niger* grown on
 147 barley flour are specialized in hydrolysing α - and β -linked galactosides from oligo-
 148 (e.g. melibiose and raffinose) and polysaccharides (e.g. xylan and galactomannan)
 149 [42,43]. Feruloyl esterase A (spot 34) removes ferulic acid from plant cell wall
 150 polysaccharides, and is known to act synergistically with xylanolytic enzymes and
 151 facilitate access to the backbone of cell wall polymers. Endo-1,4- β -xylanases cleave
 152 glycosidic bonds in the xylan backbone generating substituted or unsubstituted xylo-
 153 oligosaccharides (XOS), while β -xylosidases cleave these products from the non-
 154 reducing end, liberating xylose [44,45]. It has been reported that these key enzymes
 155 are regulated at the transcriptional level by the activator XlnR and the genes encoding
 156 the xylanolytic enzymes are induced upon growth on XOS [46]. We also found α -
 157 glucosidase AglU (GH31, spots 1, 74–76), which hydrolyses α -1-6 bonds found in
 158 oligosaccharides such as melibiose and raffinose produced by α - and β -amylases.
 159 Cellulolytic enzymes constituted another major group, including α -1,3-glucanase
 160 (mutanase, spot 78), β -1,4-glucanase (cellulase, GH12, spots 11–15, 44), β -1,4-glucan
 161 cellobiohydrolase B (GH7, spot 86), exoglucanase CBHII (GH7, spot 84) and β -

glucosidase (GH92, spots 95, 98). The polysaccharides, cellulose and hemicellulose xylan, are the major structural components of plant cell walls, and both xylanolytic and cellulolytic enzymes work in concert in degradation of cellulose to glucose. Starch degrading enzymes, i.e. glucoamylase (GH15, spots 2 and 32) and α -amylase (GH13, spot 85) as well as polysaccharide hydrolyzing α -mannosidase (GH92, spot 70), β -mannosidase MndA (GH2, spot 65) and α -galactosidase C and D (melibiase, GH36, spots 72, 99 and GH27 spot 93), were identified in the culture medium of *A. niger* grown on WAX or barley flour. In addition, β -galactosidase LacA (GH35, spot 68) that hydrolyzes lactose to galactose and glucose, was also found in both media (Fig. 3, Table 5). The most abundant proteins identified on the 2D-gels were arabinofuranosidases and xylanases present in multiple spots with varying pI values, which could be due to post translational modifications or existence of closely related gene products/isoforms, illustrating the strengths of 2DE-based studies. It was however not possible on the basis of the MS data obtained to determine the nature of the modification. Collectively, plant cell wall degrading enzymes, also termed pathogenicity/virulence factors, have been predicted to function in the penetration and maceration of plant tissues for nutrient acquisition [14]. The enzymes α/β -xylosidase, α/β -glucuronidase and feruloyl esterase, were only identified from cultures grown on WAX. Notably, cellulases and α -amylases were produced in much higher amounts by *Aspergillus* grown on barley flour, of which almost 90% is starchy endosperm [47]. Furthermore, peptides derived from spot 87 (barley flour) matched glucoamylase catalyzing breakdown of α -(1,4)-linked malto-oligosaccharides to glucose.

Growth on barley flour resulted in identification of several peptidases including tripeptidyl-peptidase, carboxypeptidases S1 and CpdS, probably involved in nutrient acquisition as well as in enhancement of fungal pathogenicity [48,49]. Moreover,

proteases have been reported to be involved in infection processes in fungi, such as *Aspergillus fumigatus* and *Candida albicans*, in plants as well as in animal hosts [49,50]. Mycelial catalase Cat1 (oxidoreductase, spots 73 and 100) can remove reactive oxygen species and protect the cells from oxidative damage. Glutaminase (GtaA, spot 90) that catalyzes hydrolysis of glutamine to glutamate and ammonia, was also identified for *A. niger* growing on barley flour.

A. niger is a well-studied filamentous fungus due to its high secretory capacity and value for biotechnology. Only a few proteome studies are reported of secreted fungal proteins on different substrates and no studies have been performed of *A. niger* on WAX and barley flour mimicking the natural hosts of cereal fungi. **Comparison and analysis of** the intra- and extra-cellular proteins produced by *A. niger* grown on xylose or maltose **showed considerable similarities in the intracellular proteomes, while** the secretomes were strongly influenced by the carbon source [13]. The secretome of the xylose-grown *A. niger* contained a variety of plant cell wall degrading enzymes, with xylanase and ferulic acid esterase being the most abundant. A comparison of our dataset of *A. niger* grown on WAX with the xylose-grown cultures [13] revealed a large overlap in the identified proteins with a few exceptions, such as α -galactosidase C (melibiase), which catalyse the hydrolysis α -1,6-linked galactose residues from oligomeric (e.g. melibiose and raffinose) and polymeric (e.g., xylan galactomannan) compounds [51]. This is expected since the backbone of arabinoxylan is a xylan composed of xylose units, but the mono- or double arabinosyl substitutions require specific enzymes for liberation. The enzyme β -glucuronidase (*A. niger*) was identified in our dataset, **while the yeast homologue and** the cell wall protein PhiA essential for phialide and conidium-spore development, was only found in cultures grown with xylose [13]. β -mannosidase (spot 65) and xyloglucanase (spot 64) were only identified

when grown on barley flour. Evidently, discrepancies are found when comparing several datasets, due to differences in the experimental designs, procedures and culture conditions (e.g. complex medium and substrate concentration).

4.4 Profiling the 2D-secretome of *Fusarium poae* grown on barley and WAX

Fusarium poae has been reported as one of the most frequent *Fusarium* species isolated from cereal grains in Finland, Japan, Norway and Sweden [52–54]. *F. poae* is a pathogenic filamentous fungus reported to produce several mycotoxins, including trichothecenes, such as deoxynivalenol, nivalenol and fusarenone-X [55–57] and to inhibit mitochondrial function and protein synthesis [58]. Little work has been performed on *F. poae*, despite its pathogenicity and hazard imposed to human health. In the present study, secretome analysis of *F. poae* grown on either WAX or barley flour showed lower protein content compared to *A. niger* cultures, despite the same amount of spores used for inoculation. The difference may be due to different growth rates and secretory capacity of the two fungi. Notably, the protein content is influenced by several factors, including inoculum size and composition of fungal mycelium. Secretome of *F. poae* gave different profiles when grown on WAX and barley flour (Fig. 3). Thirty proteins were identified by mass spectrometry (Table 5). On WAX, fungal endo-1,4- β -xylanase (GH11, spot 52) and chitinase (GH18, spot 48) were identified, while due to the lower protein content the secretome of *F. poae* grown with barley flour was dominated by plant proteins, and only three spots were found to contain fungal proteins: endo-1,4- β -xylanase A (GH10, spot 12), ubiquitin (spot 34) and a hypothetical protein (FG04936.1, spot 11) displaying homology to aminopeptidase Y. Noticeably, xylanases of family GH11 were detected in the

secretome of *F. poae* growing on WAX, while a GH10 member was detected when grown on barley flour. It has been reported that xylanases of GH11 are more efficient than the GH10 in hydrolysis of wheat bran and display two-fold higher affinity for wheat bran and 6-fold turnover rate [59]. Xylanases of GH11 are known to have a lower catalytic versatility than GH10 and preferentially cleave unsubstituted regions of arabinoxylan, whereas GH10 xylanases have broader substrate specificity and hydrolyse the AX main chain within decorated regions.

It is clear from the SDS-PAGE secretome profiles (Fig. 2) that different *Fusarium* species exhibit distinctly different protein patterns. Thus the secretome pattern of *F. graminearum* (lanes W5 and F5) differs from that of *F. poae* (lanes W8 and F8). Further optimisation and analysis of the *F. poae* secretome therefore holds promise for identification of proteins with specific roles in *F. poae* pathogenicity.

5. Conclusions

The present study provides an overview of the fungal community on barley grains, their secreted proteins and xylanolytic activities. The fungi isolated from barley grains have secretomes reflecting their enzymatic potential, which varies according to species and growth substrate. Analysis of one well-characterized (*A. niger*) and one poorly characterized (*F. poae*) fungus grown on barley flour and WAX enabled identification of new proteins, including enzymes involved in cell wall degradation and carbohydrate catabolism. This approach can provide valuable insight into secretory capacity and pathogenicity of the studied organisms as well as the molecular interactions between fungi and host plant.

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Figure captions

Fig. 1. Plate zymograms of culture supernatants (5 µL) from fungi grown on barley flour (f) and wheat arabinoxylan (w). Numbers refer to fungal isolates in Table 1.

Fig. 2. SDS-PAGE of culture supernatants from fungal isolates (Table 1) grown on barley flour (F) and wheat arabinoxylan (W) (20 µg protein). The gel was stained with Coomassie blue and numbered bands were excised for identification by MALDI-MS (Table 3).

Fig. 3. 2D-gel electrophoresis of the secretome (50 µg) of *Aspergillus niger* (A and B) and *Fusarium poae* (C and D) grown in medium containing wheat arabinoxylan (A and C) and barley flour (B and D) as sole carbon source. The numbered spots were selected for analysis by mass spectrometry. Spots a1-a105 (*A. niger*) and f1-f55 (*F. poae*) were excised for identification by MALDI-TOF/TOF MS (Table 5; Supplementary Table S1). Molecular mass markers and pI range are indicated.

Figure 1

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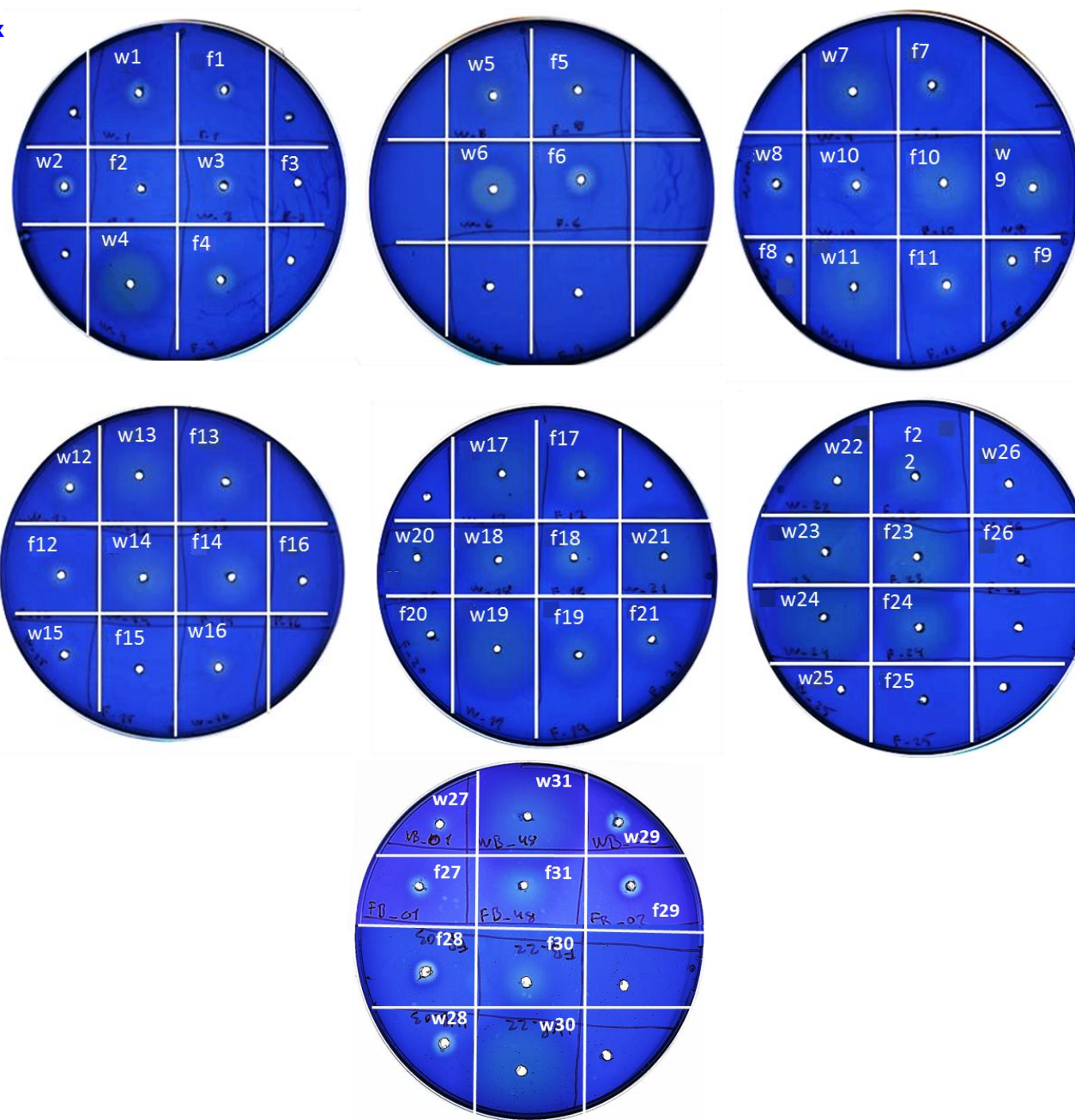


Figure 2

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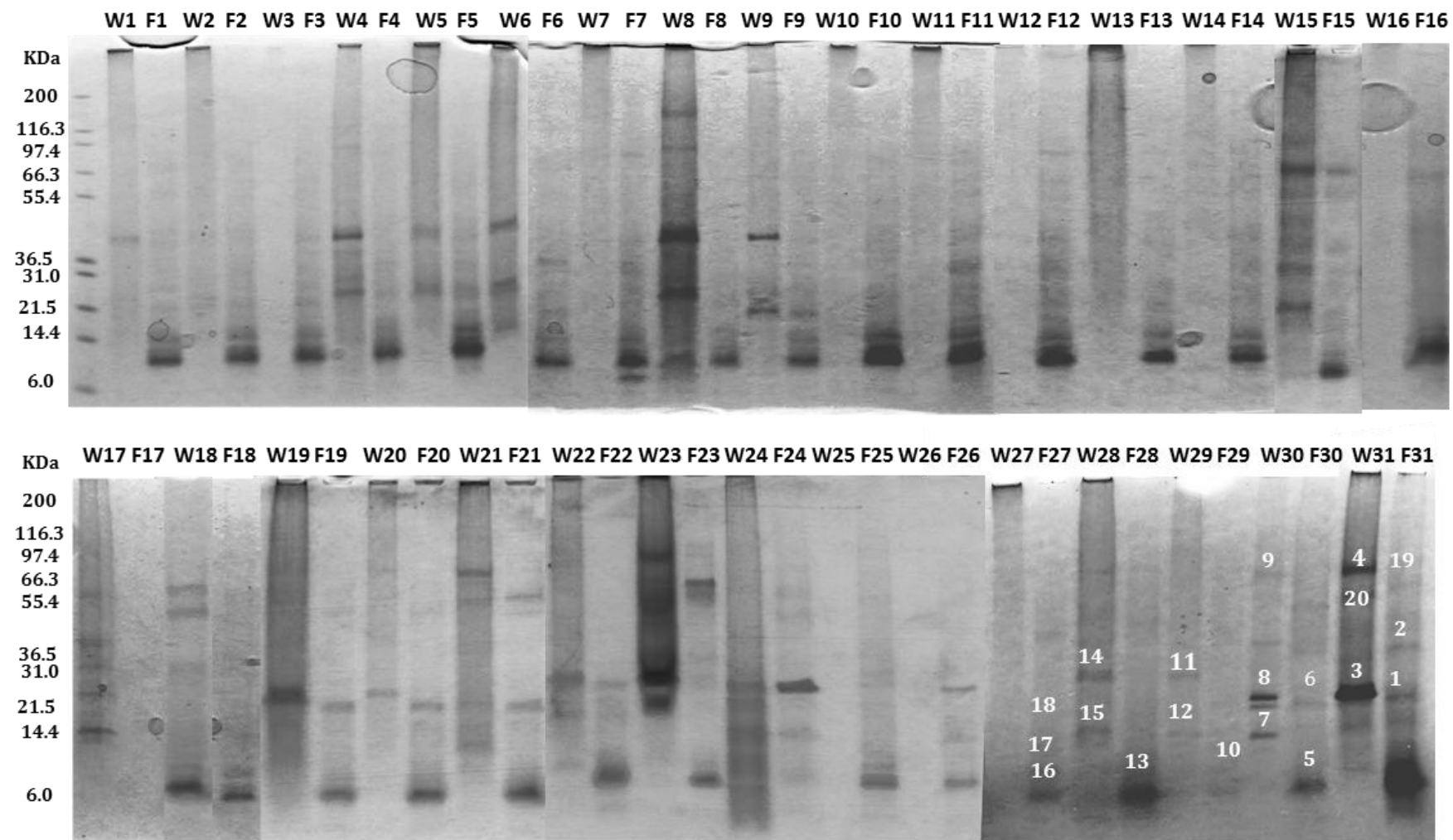
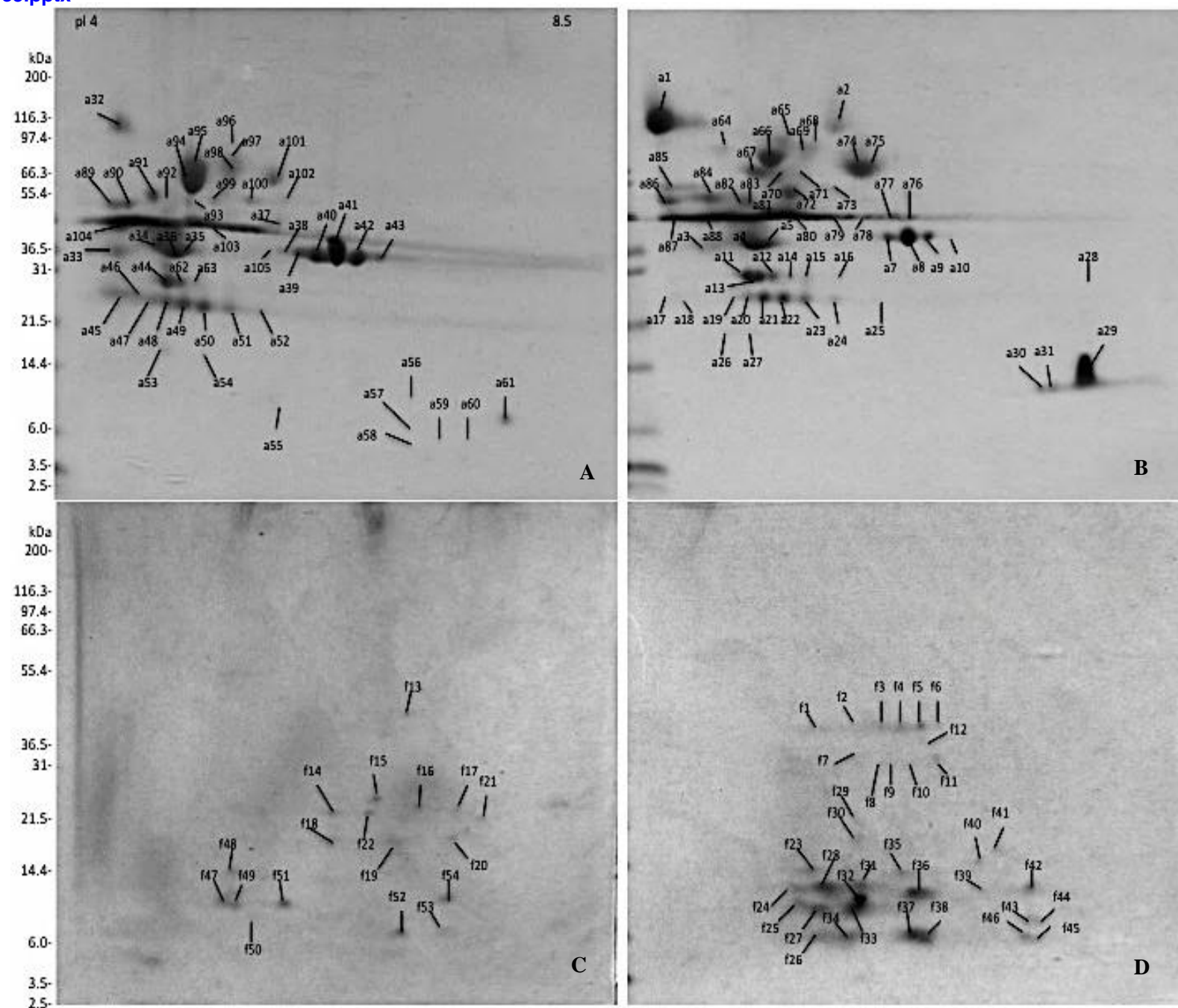


Figure 3

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